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Doxycycline alters the expression of inflammatory and immune-related cytokines and chemokines in human endometrial cells: implication in irregular uterine bleeding*

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BACKGROUND: Increased production of pro-inflammatory mediators is considered central in the manifestation of events leading to irregular uterine bleeding in progestin-only contraceptive users. Evidence suggests that in addition to its antimicrobial property, doxycycline (Dox) acts as an anti-inflammatory agent mainly through the suppression of pro-inflammatory mediators. METHODS: We tested this hypothesis in the endometrial environment using an *in vitro* model consisting of isolated human endometrial glandular epithelial and stromal cells and a human endometrial surface (HES) epithelial cell line cultured under defined conditions. RESULTS: We found that Dox at doses ranging from 1 to 100 μg/ml had a limited growth-inhibitory effect on these cells, whereas Dox in a dose-dependent manner inhibited the production of tumour necrosis factor-α (TNF-α). Using multiplex cytokine/chemokine protein analysis to test a broader range of Dox activity, we found that Dox at 25 μg/ml either alone or in the presence of 17β-estradiol (E_2), medroxyprogesterone acetate (MPA) and E_2 + MPA (10^{-8} M) as well as TNF-α (25 ng/ml), representing the endometrial environment exposed to contraceptives as well as inflammatory conditions, respectively, altered the production of multiple cytokines and chemokines as compared with untreated controls. These actions of Dox occurred in cell-, ovarian steroid- and cytokine/chemokine-dependent manners. Although Dox reduced the regulatory action of steroids on the production of these cytokines/chemokines, it was less effective on TNF-α-treated cells. CONCLUSIONS: The results support the hypothesis that Dox, by modulating the endometrial expression of multiple inflammatory-related cytokines/chemokines in a cell- and cytokine/chemokine-dependent manner, may have a therapeutic potential in patients experiencing irregular uterine bleeding, in particular in progestin-dominant contraceptive users.

Key words: chemokines/cytokines/doxycycline/endometrium/uterine bleeding

Introduction

Progesterone-dominant and progestin-only pills/implants/injectables are proven as highly effective contraceptives. However, a major drawback of these contraceptives, in particular progestin-only implants and injectables, is their associated side effects, specifically disruption of cyclic uterine bleeding (Dunn *et al*., 2001; Munro, 2001). Irregular uterine bleeding/spotting in these contraceptive users occurs mostly during the first year, with considerable improvement with increased duration of use, and treatment strategy has been largely empiric and consisted of using ethinyl estradiol (EE), ibuprofen, oral contraceptives and levonorgestrel (Archer *et al*., 1996; Burkman *et al*., 2001; Cameron, 2001; Munro, 2001). Although these treatments often result in a reduction in the number of bleeding/spotting

days, they do not provide a cure, and irregular bleeding remains the major cause of discontinuing these devices.

Endometrial environment during normal menstrual and irregular uterine bleeding has been characterized by an increased infiltration of the number of leukocytes and mast cells and alterations in endometrial tissue integrity with apparent matrix degradation (Lau *et al*., 1999; Macpherson *et al*., 1999; Hickey and Fraser, 2000; Jones and Critchley, 2000; Vincent and Salamonsen, 2000). Tissue trafficking of inflammatory/immune-related cells and tissue repair are highly regulated events and influenced by many pro-inflammatory mediators and proteases. Several pro-inflammatory mediators, proteases and angiogenic factors known to regulate these processes are expressed in human endometrium throughout the menstrual cycle (Hickey *et al*., 1999; Chegini and Williams, 2000; Critchley *et al*., 2001; Chegini *et al*., 2002; Kelly *et al*., 2002; Salamonsen *et al*., 2002). However, altered endometrial expression of some of these factors immediately before, and during, menstruation and in progestin-dominant contraceptive users is considered, at least in part, to account for normal menstrual and irregular uterine bleeding (Critchley *et al*., 2001; Kelly *et al*., 2001; Salamonsen *et al*., 2002; Jones *et al*., 2005). Although normal menstrual bleeding coincides with declining ovarian steroid production at the end of the luteal phase, accumulated evidence indicates that the endometrial expression of some of these mediators, proteases and angiogenic factors is under the regulatory control of ovarian steroids.

Doxycycline (Dox), a member of the tetracycline family, is a broad-spectrum antibiotic effective against a wide range of gram-positive and gram-negative organisms. In recent years, Dox has also been studied extensively in human and animal diseases that are characterized by high levels of pro-inflammatory mediators and protease activities (Baxter *et al*., 2002; Lamparter *et al*., 2002; Islam *et al*., 2003; Brown *et al*., 2004; Lee *et al*., 2004a,b; Onoda *et al*., 2004; Preshaw *et al*., 2004; Siemionsma *et al*., 2004; Smith and Cook, 2004; Salvi and Lang, 2005). In humans, these include osteoarthritis, rheumatoid arthritis, adult periodontitis and non-infected corneal ulcers and in models of angiogenesis where pro-inflammatory mediator and protease activities are known to be required. In each of these disorders, the beneficial effects of Dox or other tetracycline analogues are not due to their antimicrobial effects but rather their efficacy in inhibiting pro-inflammatory and protease activities.

We hypothesized that Dox, acting through this pathway, influences the endometrial pro-inflammatory cytokine/chemokine production, thus altering the outcome of irregular uterine bleeding/spotting. To test this hypothesis, we used an *in vitro* model consisting of endometrial glandular epithelial and stromal cells isolated from human endometrium and a human endometrial surface (HES) epithelial cell line cultured under defined conditions. Using this model, we first determined the potential cytotoxic effect and anti-inflammatory property of Dox by examining its action on the growth and production of tumour necrosis factor-α (TNF-α) by these cells, respectively. We then examined the effect of Dox alone or in combination with 17β-estradiol (E_2), medroxyprogesterone acetate (MPA) and E_2 + MPA as well as TNF-α, representing the endometrial environment exposed to contraceptives as well as inflammatory conditions, respectively, on the production of multiple cytokines and chemokines with a broad spectrum of inflammatory/immune-related activities.

Materials and methods

Endometrial glandular epithelial cells (GEC) and endometrial stromal cells (ESC) were isolated from portions of endometrial tissues. The tissues were obtained from premenopausal women, ranging in age from 21 to 39 years, who were undergoing hysterectomy for medically indicated reasons (excluding endometrial cancer and leiomyoma) at the University of Florida-affiliated Shands Hospital. These patients were not under any hormonal treatments at the time of surgery. The tissues were collected after obtaining approval from the University of Florida Institutional Review Board. The isolated GEC and ESC were cultured in Dulbecco's modified Eagle's medium–Ham's F-12 supplemented with 10% fetal bovine serum (FBS) (Cellgro, Mediatech, Herndon, VA, USA), and their purity was determined when freshly prepared and after the first passage by immunostaining for cytokeratin (epithelial), vimentin (stromal) and a smooth muscle actin (smooth muscle) using their various antibodies (Roberts *et al*., 2005). The cells were then cultured in 75-mm flasks in the presence of 10% FBS until reaching visible confluence and used for these experiments during their first to third passages. The HES cell line was kindly provided by Dr D. Kniss (Ohio State University, Columbus, OH, USA) and cultured as previously described (Luo *et al*., 2004).

All the materials for cell culturing, DNA synthesis and cell-proliferation assay were purchased from Fisher Scientific (Pittsburg, PA, USA), Amersham-Pharmacia Biotech (Piscataway, NJ, USA) and Sigma Chemical (St Louis, MO, USA), respectively. E_2, MPA and Dox and charcoal-stripped fetal calf serum (FCS) were purchased from Sigma and Hyclone (Logan, UT, USA), respectively. Recombinant TNF-α and TNF-α and IP-10 enzyme-linked immunosorbent assay (ELISA) kits with mean detection limits of 1 and 1.67 pg/ml, respectively, were purchased from R&D System (Minneapolis, MN, USA). Multiplex cytokine/chemokine kits were purchased from R&D System and UpState (Charlottesville, VA, USA).

Effect of Dox on ^{3}H-thymidine incorporation and cell proliferation

ESC and GEC were cultured in 48-well microplates at a density of 2.5×10^4 cell/well in the presence of 10% FBS for 24 h. The cells were washed and maintained under a serum-free condition for 24 h and then exposed to media containing 2% FBS in the presence and absence of various concentrations of Dox (1–100 μg/ml) and 2 μCi/ml ^{3}H-thymidine. The rate of ^{3}H-thymidine incorporation into the DNA was determined after 24 h of incubation (Roberts *et al*., 2005). The effect of Dox on cell proliferation was determined by 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay using ESC cultured at 2.5×10^3 cell/well in 96-well dishes and treated as described for ^{3}H-thymidine incorporation for 48 h.

Effect of Dox on TNF-α production

TNF-α, a well-established pro-inflammatory cytokine, is expressed at elevated levels in the endometrium during menses and irregular uterine bleeding. To determine whether Dox therapy influences endometrial TNF-α action and to establish a baseline for Dox action in our *in vitro* study, we first used isolated ESC. ESC were cultured in 24-well plates at a density of 2.5×10^5 cell/well in the presence of 10% charcoal-stripped FCS and phenol red/serum-free condition until reaching 70–80% confluence. The cells were maintained under a serum-free/phenol red-free condition for 24 h and then exposed to media containing 2% charcoal-stripped FCS in the presence of various

concentrations of Dox (1–50 μg/ml) for 24 h. The culture-conditioned media were collected, and their TNF-α content was determined using ELISA (Roberts *et al*., 2005).

The effect of Dox on cytokine/chemokine production

Parallel experiments were performed to determine the effect of Dox either alone or in combination with E_2, MPA and E_2 + MPA on inflammatory/immune-related cytokine and chemokine production by endometrial cells. ESC, GEC and HES cells were cultured as above describing TNF-α production. The cells maintained under a serum-free/phenol red-free condition for 24 h were treated with Dox (25 μg/ml), E_2 (10^{-8} M), MPA (10^{-8} M), E_2 + MPA or their combinations and added to phenol red-free medium containing 2% charcoal-stripped FCS. These cells were also treated with TNF-α (25 ng/ml) in the presence or absence of 25 μg/ml of Dox. The cells were incubated for 24 h, and the conditioned media were collected from treated and untreated controls and subjected to multiplex cytokine/chemokine assay according to the manufactures' protocols. Briefly, 100 μl of conditioned media in duplicates was subjected to multiplexing in 96-well configured plates in an ELISA-based sandwich principle. The assay's solid phase consists of fluorescent beads covalently linked with cytokine/chemokine-specific monoclonal antibodies, allowing the capture of each cytokine/chemokine, and corresponding biotinylated antibodies complete the reaction, which is detected with streptavidin–phycoerythrin, and the intensity is measured on Luminex 100 IS system (Luminex, Austin, TX, USA). Because the production of all the cytokines and chemokines assayed by multiplex in our study has already been established and confirmed by many other investigators, to provide an example for the validity of multiplexing, we assayed the conditioned media of TNF-α-treated cells with and without the presence of Dox for IP-10 production using ELISA.

Statistical analysis

The results are presented as mean ± SEM of three independent experiments performed in duplicate and were statistically analysed using Student's *t*-test and analysis of variance with Tukey where appropriate, with P values <0.05 considered significant.

Results

The effect of Dox on cell growth

Dose–response experiments indicate that Dox at 0.1–100 μg/ml had no significant inhibitory effect on the rate of ^{3}H-thymidine incorporation into ESC except at doses ≥50 μg/ml compared with untreated control cells (Figure 1). The rate of ^{3}H-thymidine incorporation into DNA was inhibited about 30% by Dox at 100 μg/ml compared with control (Figure 1A). Dox at these concentrations had no significant effect on the rate of cell proliferation determined by MTT cytotoxic assay as compared with untreated control (Figure 1B). A similar result was also obtained using GEC and HES cells (data not shown).

The effect of Dox on TNF-α production

Treatment of ESC with Dox dose dependently reduced the level of TNF-α production compared with untreated control cells during a 24-h period, with maximal inhibition occurring at Dox ≥25 μg/ml, with about 50% lower TNF-α production (P < 0.05; Figure 2). On the basis of these results, in further experiments we selected 25 μg/ml of Dox for the analysis of other cytokine/chemokine production by ESC, GEC and HES.

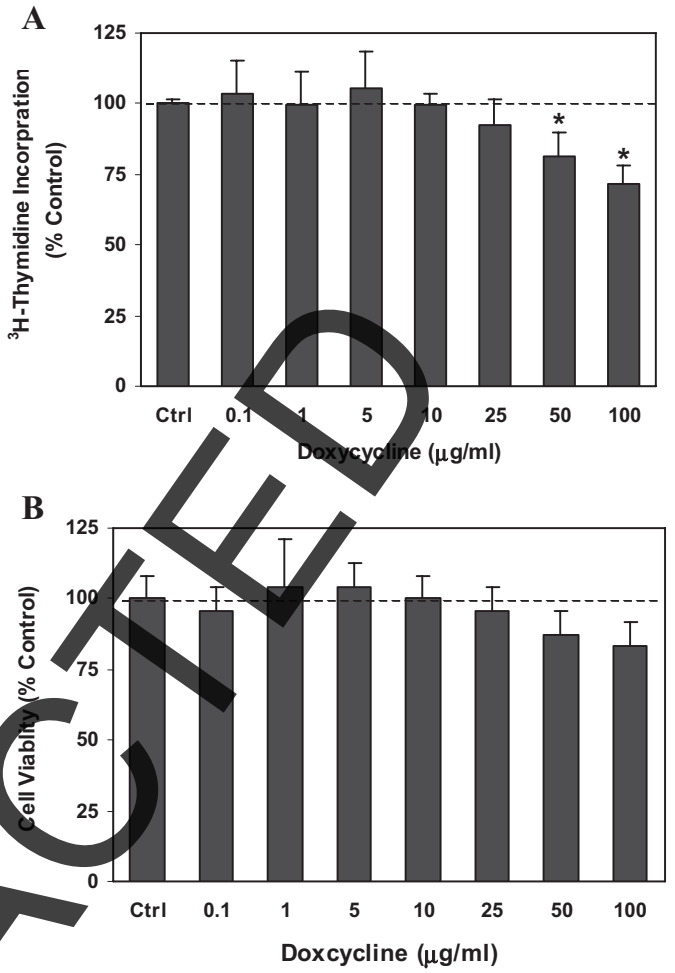

Figure 1. The effect of doxycycline (Dox) at various concentrations on the rate of ^{3}H-thymidine incorporation (**A**) or cell proliferation (**B**) on endometrial stromal cells incubated in the presence of 2% fetal bovine serum for 24 h (^{3}H-thymidine incorporation) or 48 h (cell proliferation), respectively. Note that Dox treatment had a limited effect on the rate of ^{3}H-thymidine incorporation into these cells and cytotoxicity (MTT assay) at doses <25 μg/ml but inhibited these parameters at higher doses. The bars represent mean ± SEM of triplicate experiments using endometrial stromal cells (ESC) isolated from three different tissues. Asterisks denote statistically different from untreated control (Ctrl), P < 0.05.

The effect of Dox on cytokine/chemokine production

Several pro-inflammatory modulators are considered to participate in the events leading to irregular uterine bleeding. We examined the effect of Dox alone or in combination with ovarian steroids on the production of several key pro-inflammatory cytokines and chemokines using endometrial cells. The ESC, GEC and HES cells cultured under a defined condition produced various levels of 25 cytokines, chemokines and growth factors assayed, including interleukin-1α (IL-1α), IL-1β, IL-2 to IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), interferon-γ (IFN-γ), MIP-1α, MIP-1β, eotaxin, IP-10, monocyte chemotactic protein-1 (MCP-1) and regulated on activation and normally T-cell expressed and presumably secreted (RANTES).

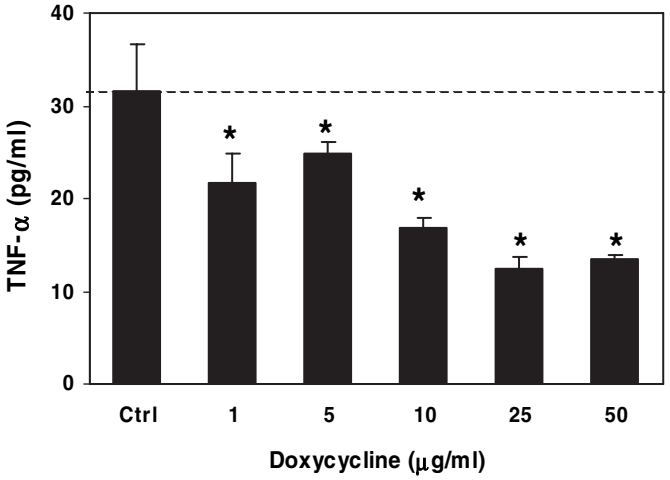

Figure 2. The bar graphs show the dose–response effect of doxycycline (Dox) on tumour necrosis factor-α (TNF-α) production detected in culture-conditioned media of endometrial stromal cells. The cells were treated with Dox at 1 and 50 μg/ml for 24 h, and the culture-conditioned media from treated and untreated controls (Ctrl) cells were collected and subjected to enzyme-linked immunosorbent assay. The results are the mean ± SEM from three independent cell cultures prepared from different tissues and performed in duplicate. Asterisks denote statistically different from their various untreated controls ($P < 0.05$).

These cells produced low- (<20 pg/ml) to-undetectable levels of IL-1β, IL-2 to IL-5, IL-7, IL-10, IL-12p40, IL-12p70, IL-17, GM-CSF, G-CSF, IFN-γ, MIP-1α, MIP1-β and eotaxin, and their levels did not significantly change following treatments with Dox, E_2, MPA and their combinations. Therefore, further experiments and analyses were performed using IL-1α, IL-6, IL-8, IL-15, MCP-1, RANTES and IP-10.

As shown in Figure 3A–F, ESC, GEC and HES produced variable levels of IL-6 (A), IL-8 (B), IL-15 (C), MCP-1 (D), RANTES (E), IP-10 (F) and IL-1α (data not shown). The levels of these cytokines and chemokines in most cases increased following treatment of ESC, GEC and HES with E_2, MPA and their combinations as compared with untreated controls, with inhibition of IL-6 and IL-8 production in GEC and HES by MPA (Figure 3). In contrast, treatment of ESC, GEC and HES with TNF-α (25 ng/ml), representing an inflammatory condition, resulted in a significant increase in the production of IL-6, IL-8, IL-15, MCP-1, RANTES and IP-10 ($P < 0.05$; Figure 3). We found that Dox (25 μg/ml) either was without effect or, in a cell-specific manner, significantly inhibited and/or stimulated IL-6, IL-8, IL-15, MCP-1, RANTES and IP-10 production in ESC, GEC and HES (Figure 3). In addition, Dox co-treatment with E_2, MPA and E_2 + MPA reduced their actions on IL-6, IL-8, IL-15, MCP-1, RANTES and IP-10 production by these cells; however, the action of Dox occurred in a cell-, steroid- and cytokine/chemokine-dependent manner (Figure 3). Similarly, co-treatment of ESC, GEC and HES with Dox and TNF-α reduced the production of IL-6, IL-8, IL-15, MCP-1, RANTES and IP-10 by these cells, also in a cell- and cytokine/chemokine-dependent manner (Figure 3). The levels of IP-10 detected in the culture media from cells incubated with TNF-α, either alone or in the presence of Dox, assayed individually by ELISA showed comparable results to IP-10 assayed by multiplexing, regarding both levels of IP-10 detected and effects of TNF-α and Dox on IP-10 production (data not shown).

Discussion

In recent years, Dox has been studied at subantimicrobial concentrations in human and animal diseases that are characterized by high levels of pro-inflammatory mediators and protease activities, the diseases including osteoarthritis, rheumatoid arthritis, adult periodontitis and pelvic inflammatory disorder (Brown *et al*., 2004; Lee *et al*., 2004a,b; Preshaw *et al*., 2004; Siemonsma *et al*., 2004; Salvi and Lang, 2005). Under these conditions, Dox therapy has been shown to inhibit/reduce the local production of pro-inflammatory mediators and protease activities, resulting in an enhanced reparative process. During menstruation and in women who experience irregular uterine bleeding due to progesterone-only or progesterone-dominant contraceptives, the endometrial environment is also characterized by elevated levels of pro-inflammatory mediators and protease activity (Chegini and Williams, 2000; Critchley *et al*., 2001; Kelly *et al*., 2002; Salamonsen *et al*., 2002; Chegini *et al*., 2003; Rhoton-Vlasak *et al*., 2005). Therefore, Dox therapy may be useful for the management of irregular bleeding/spotting in women using these contraceptives. Using isolated human ESC, GEC and HES cell lines as *in vitro* model, we tested this hypothesis and demonstrated that Dox altered the production of several inflammatory-related cytokines/chemokines, without having a significant cytotoxicity, except at high doses. However, the effect of Dox on the production of these cytokines and chemokines varied from an inhibitory or stimulatory effect to no significant change. Additionally, Dox treatment altered the production of cytokines/chemokines in these cells co-treated with E_2, MPA and E_2 + MPA as well as cells treated with TNF-α, representing the endometrial environment exposed to contraceptives as well as inflammatory conditions, respectively, in a cell-, steroid- and cytokine-dependent manner. How Dox regulates the expression of these and other inflammatory mediators in endometrial cells is yet to be established; considering the cell-type and cytokine-dependent action of Dox, with differential interaction with ovarian steroids and pro-inflammatory mediators, the therapeutic benefit of Dox is likely to involve a complex molecular mechanism(s). However, the results support the hypothesis that Dox, acting independent of its antimicrobial property, regulates the endometrial cytokine/chemokine production, thereby altering their autocrine/paracrine actions that lead to endometrial inflammatory response.

Regarding the production of multiple cytokines/chemokines by the endometrial cells, we found that under the culture conditions of our study, ESC, GEC and HES produced low- (<20 pg/ml) to-undetectable levels of many of these cytokines and chemokines during the 24 h of incubation. These results are in agreement with those of previous studies that reported similar levels of production of IL-1β, IL-2 to IL-5, IL-7, IL-10, IL-12p40, IL-12p70, G-CSF, IFN-γ, MIP-1α, MIP-1β and eotaxin by ESC and a few studies using GEC (Mazzeo *et al*., 1998; Bergqvist *et al*., 2000; Hornung *et al*., 2000; Cork *et al*., 2001;

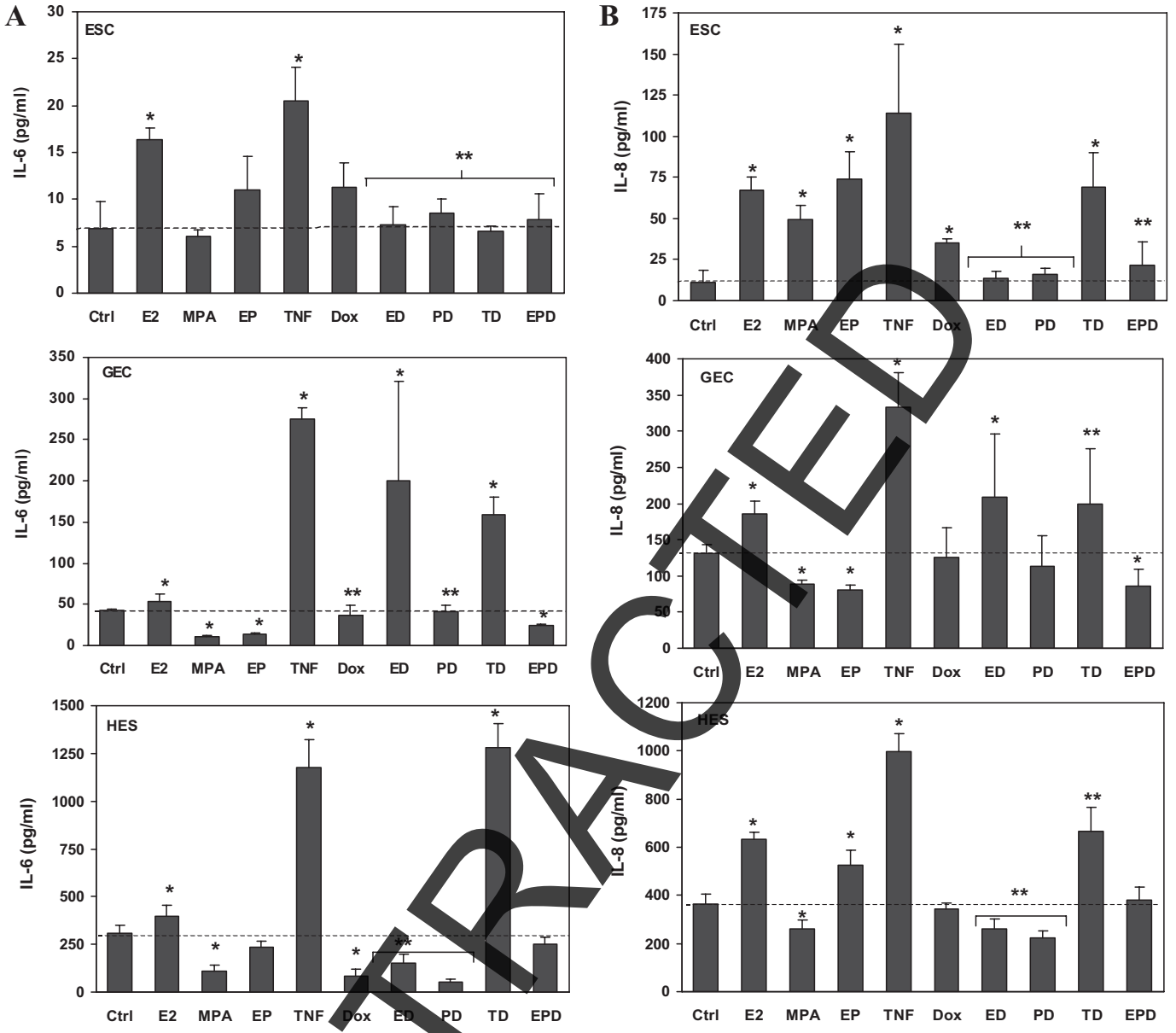

Figure 3. The bar graphs (Figs. A-F) show the levels of interleukin (IL-6), IL-8, IL-15, monocyte chemotactic protein-1 (MCP-1), regulated on activation and normally T-cell expressed and presumably secreted (RANTES) and IP-10 detected in endometrial stromal cells (ESC), endometrial glandular epithelia cells (GEC) and human endometrial surface (HES) epithelial cell line culture-conditioned media of untreated controls (Ctrl) or following treatments with doxycycline (Dox) (25 μg/ml), 17β-estradiol (E_2), medroxyprogesterone acetate (MPA), E_2 + MPA (EP) at 10^{-8} M or their combinations [ED (E_2 + Dox), PD (MPA + Dox) and EPD (E_2 + MPA + Dox)] as well as treatment with tumour necrosis factor-α (T) (25 ng/ml) in the presence [TD (T + Dox)] or absence of Dox and added to phenol red-free medium containing 2% charcoal-stripped fetal calf serum (Ctrl) for 24 h. The culture media were analysed for 25 cytokines/chemokines by multiplexing, and the results are the mean ± SEM of three independent cell cultures prepared from different tissues and performed in duplicates. In E_2, MPA, E + MPA, TNF and Dox treated cells * are statistically different as compared with untreated controls. In cells treated with Dox in combination with E_2, MPA, E + MPA and T ** is different from controls and their respective *. Lines over the bars point out the difference between E_2, MPA, E + MPA and T with their combination treatments with Dox ($P < 0.05$).

Nasu *et al*., 2001, 2003; Caballero-Campo *et al*., 2002; von Wolff *et al*., 2002; Zhao *et al*., 2002; Fahey *et al*., 2005; Luk *et al*., 2005). We also found that not all the cytokine/chemokine production assayed in our study is influenced by ovarian steroid treatments, and those that responded to E_2, MPA and E_2 + MPA treatments were regulated in a cell- and steroid-dependent manner. Several previous studies have reported the influence of ovarian steroids on the expression of some of these cytokines/chemokines such as IL-1β, GM-CSF, IFN-γ, MIP-1α, MIP1-β and eotaxin in ESC and a few in GEC; however, there is no report of their expression or regulation in HES for comparison. Regarding IL-1α, IL-6, IL-8, IL-15, MCP-1, RANTES and IP-10 production and their regulation by the ovarian steroids in ESC and GEC, in general our results are in agreement with those of previously published reports (Arici *et al*., 1999; Bergqvist *et al*., 2000; Nasu *et al*., 2001, 2003; Kai *et al*., 2002; Zhao *et al*., 2002; Roberts *et al*., 2005). We believe that any differences observed between the results of

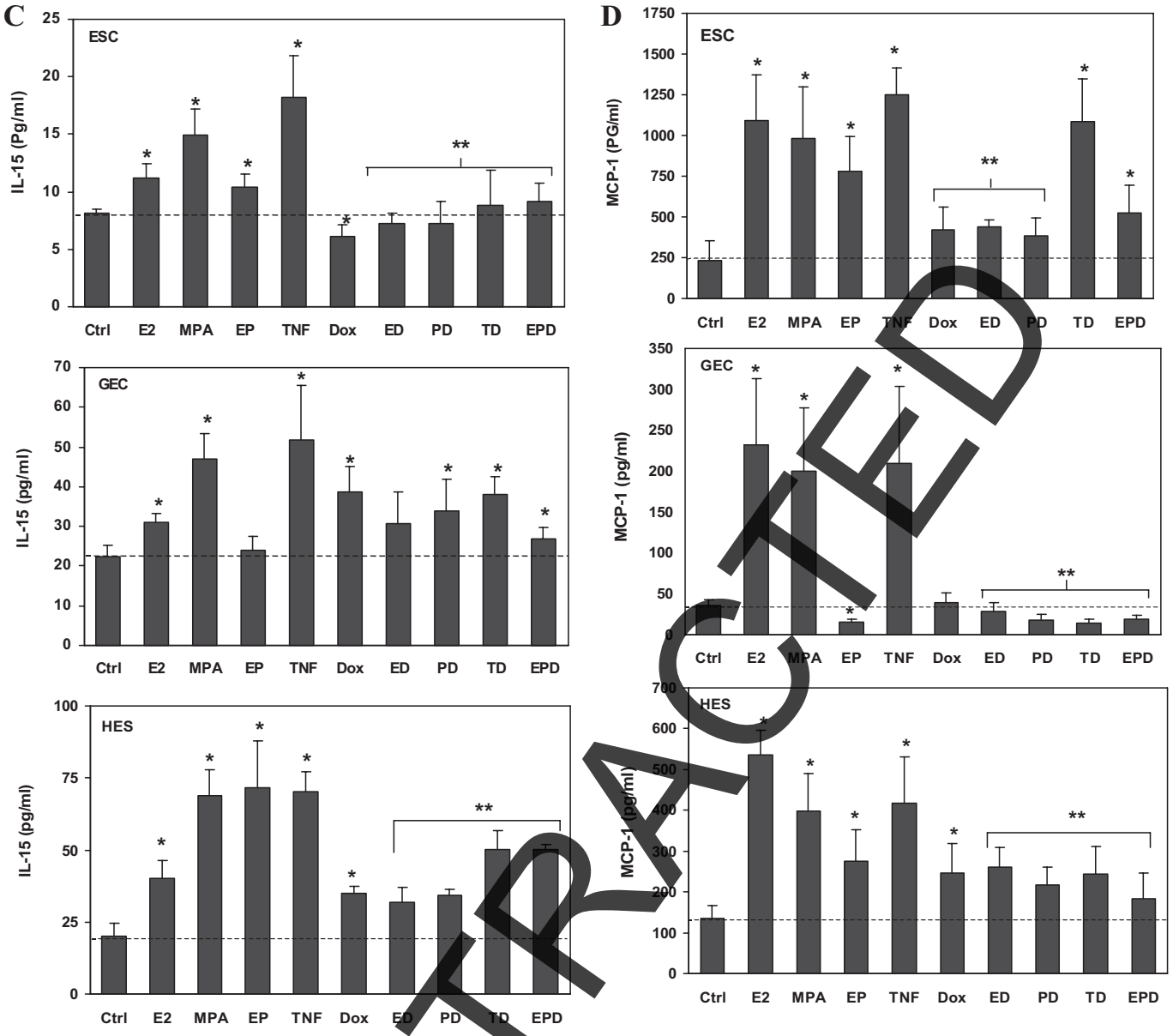

Figure 3. Continued

this study and previous observations are due to the type of assays (ELISA versus multiplexing) and the culture conditions. However, a recent study determining the production of similar cytokines in ESC reported a considerable similarity between the result obtained using ELISA and multiplexing (duPont *et al*., 2005). In addition to ovarian steroids, autocrine and paracrine actions of these cytokines and chemokines could result in the differential regulation of their own production or the production of others, which is well documented in endometrial cells under *in vitro* conditions.

Regarding the effect of Dox on the production of multiple cytokines/chemokines by the endometrial cells, it occurred at a subantimicrobial dose with a broad range of activities and targets. Dox either alone or in combination with the ovarian steroids and TNF-α altered the production of IL-1α, IL-6, IL-8, IL-15, MCP-1, RANTES and IP-10 by the endometrial cells in a cell-type, steroid- and cytokine/chemokine-dependent manner. Therefore, Dox was more effective in modulating cytokine/chemokine production by ESC as compared with GEC and HES, to an extent functioned differently during co-treatment with E_2 or MPA and displayed a more inhibitory action towards the production of chemokines as compared with cytokines, specifically following co-treatment with MPA. In contrast, Dox was less effective in modulating the effect of TNF-α on the production of these cytokines/chemokines as compared with ovarian steroid-treated cells, although Dox in a dose-dependent manner reduced TNF-α production by ESC. Because most of the endometrial breakdown during menses and irregular bleeding due to progesterone-only contraceptives occurs in the stromal compartment, the cell-specific action of Dox, specifically following co-treatment with MPA, suggests that Dox therapy may also act in compartment-dependent manner, altering the local production of these inflammatory mediators. This is particularly interesting because inflammatory and

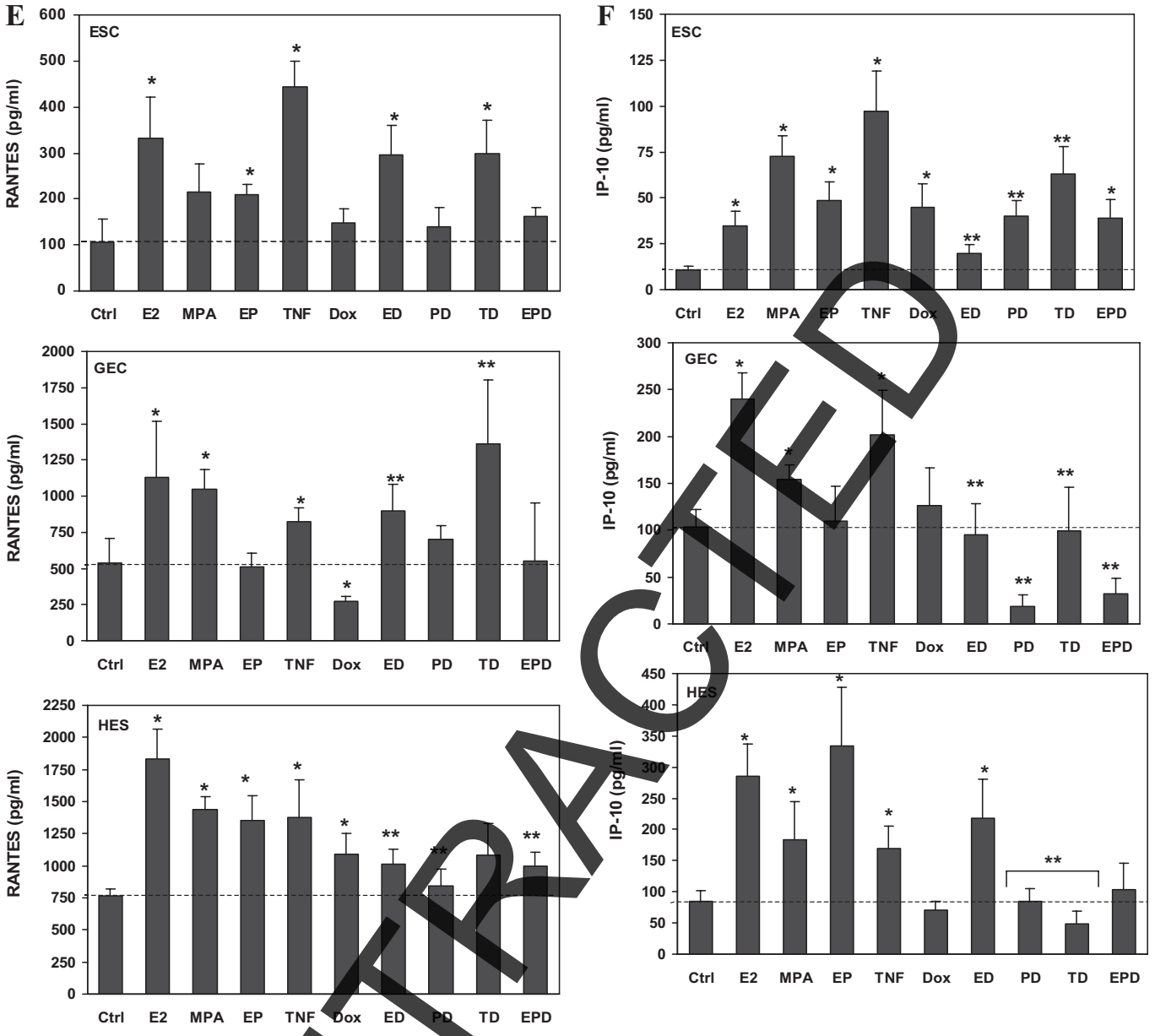

Figure 3. Continued

immune-related cells traffic through the endometrium and are localized among the ESCs in a larger number, specifically during menstruation and breakthrough bleeding/spotting. Because these inflammatory and immune-related cells are the major source of the pro-inflammatory cytokines/chemokines and proteases, reduction in their activities by Dox therapy may help reduce the endometrial tissue breakdown during breakthrough bleeding/spotting.

Alteration in endometrial microvasculature and inflammatory reaction, coinciding with infiltrating leukocytes, monocytes and mast cells, is among the key events occurring during normal menstruation and irregular uterine bleeding (Lau *et al*., 1999; Macpherson *et al*., 1999; Hickey and Fraser, 2000; Jones and Critchley, 2000; Vincent and Salamonsen, 2000). Many cytokines/chemokines examined in our study, such as IL-8, IL-15, MCP-1 and RANTES, act as key regulators of leukocyte differentiation and activation and orchestrate local tissue inflammatory/immune response. Our results with Dox action on the endometrial cells imply that in a clinical setting, Dox therapy through a similar mechanism may alter the endometrial production of these cytokines/chemokines, resulting in the stabilization of the inflammatory response during menses and in women experiencing irregular bleeding/spotting due to progesterone-only and progesterone-dominant contraceptives. However, our results with TNF-α suggest that during menses, a period associated with elevated endometrial expression of TNF-α, Dox may have a limited/reduced activity. Although the molecular mechanism(s) of how Dox regulates the endometrial production of these cytokines/chemokines is yet to be determined, a recent pilot study examining the efficacy of two treatment strategies has found that Dox at 100 mg twice daily for 5 days is equally effective as mifepristone and mifepristone plus EE in reducing the number of days

of uterine bleeding/spotting in women using a progesterone-only contraceptive (Weisberg *et al*., 2006). Interestingly, Dox at 250 mg four times daily for 4 days is routinely used in women undergoing IVF starting at 1 day before embryo transfer. No reason has been given for the beneficial use of Dox in these women under this protocol; however, Dox therapy is considered to act through antimicrobial property under this and other protocols such as pelvic inflammatory disease and premenstrual syndrome (Toth *et al*., 1988; Walters and Gibbs, 1990). We have previously observed few incidences of endometritis in Norplant users with abnormal uterine bleeding (Rhoton-Vlasak *et al*., 2005), and Dox therapy acting through both antimicrobial and anti-inflammatory properties may be beneficial, although bleeding patterns return to normal following removal of the implants. Dox either directly or through modulation of these cytokines and chemokines may also alter the expression of matrix metalloproteinases (MMPs), proteases known to regulate tissue remodelling, which is a critical event in endometrial tissue repair following menses and in patients with irregular uterine bleeding (Critchley *et al*., 2001; Kelly *et al*., 2001; Salamonsen *et al*., 2002; Chegini *et al*., 2003). In a mouse model of endometrial breakdown, with elevated production of MMPs, treatment with Dox reduced endometrial MMP, without affecting the endometrial tissue integrity (Kaitu'u *et al*., 2005).

In conclusion, these results provide evidence that Dox, acting through its anti-inflammatory property, alters the expression profile of a selective number of pro-inflammatory cytokines/chemokines in endometrial cells, supporting its therapeutic potential in women experiencing irregular uterine bleeding/spotting, in particular those associated with progestin-dominant or progesterone-only contraceptives.

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